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Mode of Action of the Azasteroid Antibiotic 15-Aza-24-Methylene-D-Homocholesta-8,14-Dien-3β-ol in *Ustilago maydis*†

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Ustilago maydis sporidia treated with 0.1 μg of azasterol (15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol) per ml appeared branched and vacuolated after 6 h of incubation. Sporidial multiplication, dry weight increase, and synthesis of protein, deoxyribonucleic acid, and ribonucleic acid were only slightly or moderately inhibited during the initial 3 h of incubation. An increase of free fatty acids was observed in lipid extracts of treated sporidia after incubation for 3 h or more. Ergosterol synthesis was completely inhibited within 1 h and there was a gradual decline of ergosterol content during 6 h which was accompanied by an accumulation of the sterol intermediate ergosta-8,14-dien-3β-ol. The results indicate that toxicity of the azasterol results from specific inhibition of the reduction of the sterol C-14(15) double bond. A triarimol-tolerant strain of *Cladosporium cucumerinum* was tolerant to the azasterol, but an imazalil-tolerant strain of *Aspergillus nidulans* was not.

The antibiotic complex A25822 consists of seven structurally similar azasterols isolated from the culture medium and mycelium of the fungus *Geotrichum flavo-brunneum* (3). The components of this complex were tested for toxicity to a variety of fungi and bacteria (10). Component A25822B (15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol) (Fig. 1A) proved to be not only the major fraction of the complex (15) but also the most active antibiotic (10). Topical application of A25822B reduced the severity of *Trichophyton mentagrophytes*-induced lesions in guinea pigs. Administration of the antibiotic orally or intraperitoneally increased the survival of mice infected with *Candida albicans*. These results indicate a potential value of A25822B in clinical chemotherapy (10).

The effect of A25822B on sterol metabolism in *Saccharomyces cerevisiae* was studied by investigators at Oregon State University (2, 11, 12). Using high concentrations (20 and 122 μM), they initially observed competitive inhibition of *S*-adenosylmethionine:Δ^{24}-sterol methyl transferase in vitro and a reduction of ergosterol in treated cells (2). At a lower concentration (0.18 μM), an accumulation of ergosta-8,14-dien-3β-ol (Fig. 1B) was observed in the treated fungus, indicating that the sterol C-14(15) reductase enzyme was inhibited (11, 12).

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These results are of interest in respect to the action of triarimol and of similar compounds which inhibit ergosterol biosynthesis in *Ustilago maydis* by interferring with sterol C-14 demethylation (17; M. J. Henry and H. D. Sisler, Phytopathol. News **12**:69, 1978). As a consequence of this inhibition, there is an accumulation of obtusifoliol, 14α-methyl-ergosta-8,24(28)-dienol, and 24-methylenedihydrolanosterol in *U. maydis*. These results are in striking contrast to the intermediate that accumulates when ergosterol biosynthesis is inhibited by A25822B. This report describes the toxic and metabolic effects of the latter compound and compares these effects with those produced by sterol C-14 demethylation inhibitors.

MATERIALS AND METHODS

Toxicant. The azasterol A25822B, 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol, was a gift of the Eli Lilly Co., Indianapolis, Ind. A stock solution (10 μg/ml) was prepared by adding 1 mg of azasterol in methanol to 100 ml of a modified medium (18) of Coursen and Sisler (6). The final concentration of methanol did not exceed 1%.

Organisms and culturing conditions. Sporidia of *Ustilago maydis* (DC.) Cda., ATCC 14826, were cultured in a liquid medium (6) containing yeast extract (2 g/liter). Logarithmically growing sporidia were washed twice with 0.04 M phosphate buffer (pH 6.6) and suspended in a modified nutrient medium (18) of Coursen and Sisler (6). Final concentration of sporidia

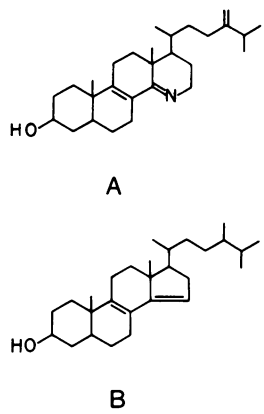

FIG. 1. *(A) Azasterol (A25822B) and (B) ergosta-8,14-dien-3β-ol.*

was equivalent to 0.4 mg of sporidial dry weight per ml.

Azasterol was added from stock solution (1.0 ml/100 ml of suspension) with the appropriate solvent controls. The cultures were incubated on a rotary shaker at 30°C.

Wild-type and a triarimol-tolerant strain of *Cladosporium cucumerinum* Ell. and Arth. (19) and wild-type and an imazalil-tolerant strain of *Aspergillus nidulans* (Eidam) Wint. (24) used for a cross-resistance study were cultured for 4 to 7 days on malt agar slants. Conidia were prepared as described by Sherald et al. (19), and about 2,000 conidia were spread on the surface of solidified medium (18) in petri plates containing various concentrations of azasterol. Toxicity was evaluated on the basis of colony development after 4 days of incubation.

RNA, DNA, and protein analysis. Ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein from *U. maydis* were extracted and quantified according to the procedures of Ragsdale and Sisler (18).

Lipid extraction and separation. Sporidia were washed and lyophilized, and the lipids were extracted with chloroform–methanol (2:1, vol/vol) using a modified Folch procedure (8). Lipid components were separated by thin-layer chromatography (TLC), using TLC plates coated with Adsorbosil-1 (Applied Science Laboratories, University, Park, Pa.) 0.5 mm thick and developed in a solvent system of hexane–diethyl ether–acetic acid (70:30:1, vol/vol/vol). Detection of components was achieved by spraying developed plates with 50% H_2SO_4 and charring in a 200°C oven.

Free sterols were isolated from TLC plates by the following procedure. Standards of lanosterol and ergosterol were spotted at the two sides of the streaked TLC plates. After development with the above solvent system, the standards were sprayed with 0.2% 2′, 7′-dichlorofluorescein (in 95% ethanol) to make them visible under ultraviolet irradiation. The area of silica gel from immediately above the lanosterol standard to that immediately below the ergosterol standard was removed, and sterols were eluted with diethyl ether.

Ergosta-8,14-dien-3β-ol was separated from other free sterols on TLC plates coated with 0.5 mm of SilicAR 7GF (Mallinckrodt, Inc., St. Louis, Mo.) containing 10% $AgNO_3$. The plates were developed in chloroform–acetone (140:1, vol/vol). The sterol was detected under short-wave ultraviolet irradiation as a quenching band at a higher R_f than that of ergosterol and was eluted with diethyl ether.

Sterol analysis. Free sterols isolated from TLC were analyzed by gas-liquid chromatography on a Glowall Chromalab 301 (with argon ionization detector) using a glass column (183 cm by 3.4 mm) packed with 100/120-mesh Gas-Chrom Q coated with 3% SE-30 (Applied Science Laboratories) and a column temperature of 250 to 259°C. Retention times were calculated relative to a value of 1.0 for cholesterol. Quantification was based on an internal standard of cholesterol.

Mass spectrographic analysis was performed using an LKB model 9000 gas chromatograph mass spectrometer (LKB Produkter AB, Stockholm, Sweden), equipped with a Varian Spectro System 100 MS Data System. The sample was introduced directly into the ionization chamber; the ionization energy was 70 eV.

Nuclear magnetic resonance determination was performed by H. Finegold (U.S. Department of Agriculture, Beltsville, Md.) with a 60 MHz JEOL FX 60-Q Fourier transform NMR using a 5-mm probe, with $CDCl_3$ as the solvent and tetramethylsilane as the internal standard.

RESULTS

General toxicity and metabolic effects. Concentrations of azasterol less than 0.05 μg/ml did not effectively inhibit growth of *U. maydis* sporidia over a 24-h period; however, concentrations above 0.25 μg/ml almost completely prevented growth. Increase in sporidial number and dry weight of cultures treated with 0.1 μg of the azasterol per ml was nearly unaffected for 2 to 3 h, but was severely curtailed thereafter (Fig. 2). Many sporidia became branched and appeared vacuolated after incubation with the toxicant for 6 to 24 h. The effect on protein, RNA, and DNA synthesis was similar to that on dry weight increase, although inhibition of RNA synthesis was somewhat more delayed (Fig. 3).

Addition of 10 μg of ergosterol per ml in ethanol to treated cultures did not alleviate the toxic effects of the azasterol on growth over a 12-h period.

Effect of azasterol on triarimol- and imazalil-tolerant fungal mutants. A triarimol-tolerant mutant of *C. cucumerinum* (19) was also tolerant to the azasterol (Table 1). When treated with azasterol, the mutant strain exhibited a narrow dosage response with a distinct cut-off point for colony development, and no tendency for colony appearance at higher concentrations with prolonged incubation. When treated with triarimol, the mutant exhibited only a progressively slower colony development with increasing concentrations up to 40 μg/ml with no reduction in the number of colonies. Neither

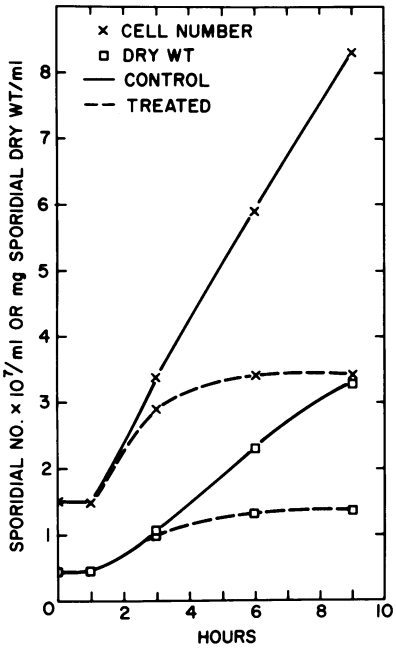

FIG. 2. *The effect of azasterol (0.1 μg/ml) on dry weight and sporidial number increase of U. maydis in liquid culture.*

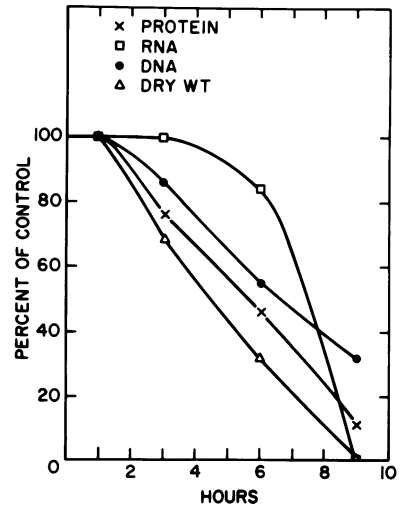

FIG. 3. *Effect of azasterol (0.1 μg/ml) on the rate of protein, DNA, and RNA synthesis and dry weight increase of U. maydis sporidia.*

compound prevented conidial germination in the mutant or wild-type strain. An imazalil-tolerant mutant of *A. nidulans* showed the same response as the wild-type strain, indicating no cross-resistance to the azasterol (Fig. 4).

TABLE 1. *Minimum concentration of azasterol (A25822B) or triarimol preventing colony development (in 4 days) from conidia of wild type and a triarimol-tolerant mutant of C. cucumerinum*

Compound	Concn (μg/ml)	
	Wild type	Mutant
A25822B	0.05	0.8
Triarimol	0.1	>40

Effect on sterols. Analysis of free sterols by gas-liquid chromatography showed that no ergosterol synthesis occurred in azasterol-treated cultures during the first hour of incubation (Fig. 5). There was in fact about a 30% decrease in free ergosterol during the first hour, but the amount remained nearly stationary thereafter. In contrast, ergosterol in control cultures increased 20% in the first hour and increased sevenfold by 6 hours.

At 1 h gas-liquid chromatographic scans of extracts from treated sporidia revealed a major sterol peak not characteristic of extracts from untreated sporidia. The component had a retention time of 1.32; it comprised 15% of the total free sterol at 1 h and 90% by 6 h (Fig. 6, peak 4). The rate of accumulation of this component in treated cultures nearly parallelled that of ergosterol in control cultures (Fig. 5). A mass spectrum of the sterol (1.32) gave a fragmentation pattern identical to that of ergosta-8,14-dien-3β-ol) (L. G. Dickson, Ph.D thesis, University of Maryland, College Park, 1971). The mass spectrum showed the following peaks with relative abundance indicated in parentheses: *m/e* 398 (100%, $C_{28}H_{46}O$, M^+), 383 (82%), 365 (41%), and 271 (23%), corresponding to a loss of CH_3, H_2O + CH_3, and the saturated side chain C_9H_{19}, respectively. The nuclear magnetic resonance spectrum was consistent with that recorded for ergosta-8,14-dien-3β-ol (personal communication with M. J. Thompson, Insect Physiology Laboratory, Plant Protection Institute, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Beltsville, Md.), as was also the absorption maximum at 251 nm (in hexane) (7).

There were differences in minor sterol components in control and treated sporidia. The sterol with a retention time of 1.42 (peak 2), previously identified as ergosta-5,7-dienol (16), was present in extracts of control sporidia but not in those from treated sporidia. The sterol with a retention time of 1.87 (peak 3), identified as 24-methylenedihydrolanosterol, is normally found in small quantities in *U. maydis* sporidia (16). The level of this sterol did not differ appreciably in control and treated sporidia. Another

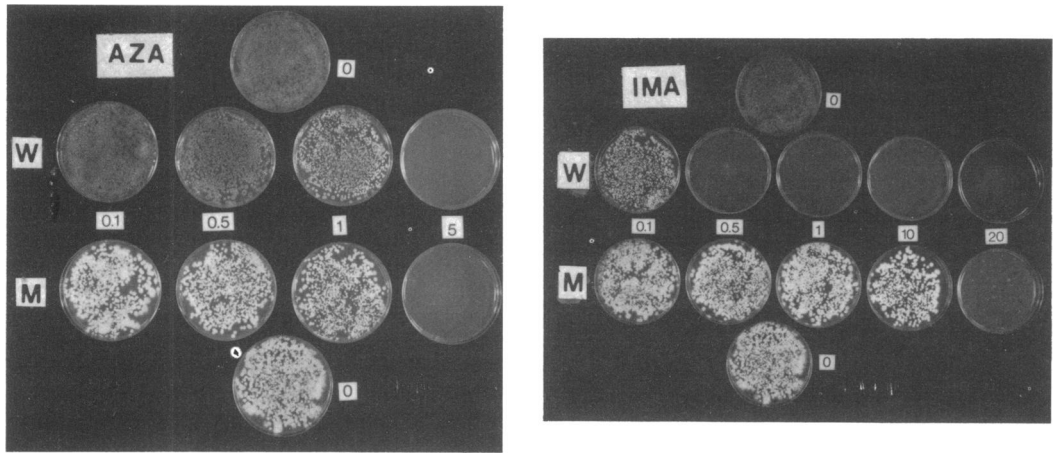

FIG. 4. *Cultures of wild-type (W) and imazalil-tolerant mutant (M) of A. nidulans treated with various concentrations (μg/ml) of azasterol (AZA) and imazalil (IMA). Cultures were incubated at 37°C for 5 days.*

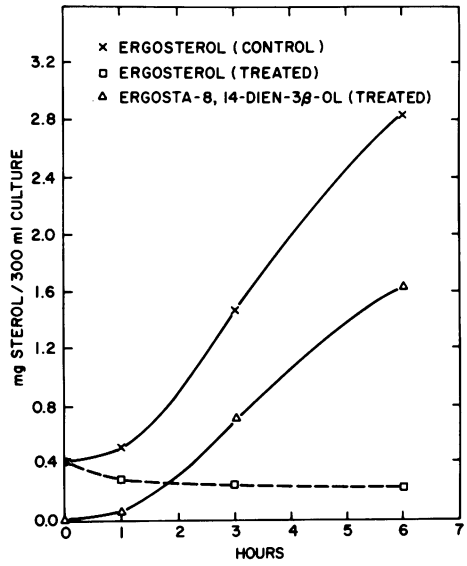

FIG. 5. *Effect of azasterol (0.1 μg/ml) on the increase of free ergosterol in treated and control cultures of U. maydis and of free ergosta-8,14-dien-3β-ol in treated cultures.*

sterol (peak 5), identified on the basis of gas-liquid chromatographic retention time (1.48) and TLC chromatographic characteristics as obtusifoliol, was detected only in treated cells. The sterol was found only in trace amounts at all time intervals between 1 and 6 h after treatment.

TLC analysis of the total lipid fraction revealed an increase of compounds which migrated to the region of free fatty acids in treated sporidia grown for 3 h or more. There was no apparent effect on the levels of sterol esters or triglycerides.

DISCUSSION

The effects of the azasterol antibiotic A25822B on the growth of *U. maydis* resemble those of other inhibitors of ergosterol biosynthesis (4, 5, 17, 21). Ergosterol biosynthesis was strongly inhibited within the first hour, whereas dry weight, cell number, and synthesis of protein and nucleic acids were unaffected or inhibited much less than ergosterol biosynthesis for nearly 3 h. The azasterol produces a branching morphology of *U. maydis* sporidia, strong inhibition of hyphal growth of *C. cucumerinum* without appreciable effect on conidial germination, and accumulation of free fatty acids in *U. maydis* sporidia after several hours of incubation. These effects are characteristic for the group of ergosterol biosynthesis inhibitors, which includes such compounds as triarimol, triforine (17), imazalil (21), and fenarimol (5).

The absence of any net ergosterol synthesis during the first hour after treatment with 0.1 μg of the azasterol per ml indicates that the sterol pathway is very effectively blocked. The reason for the apparent loss of free ergosterol during this period is not known. One explanation may be a depletion resulting from a continued esterification of ergosterol without concomitant synthesis.

In *U. maydis* the removal of the C-14 methyl is probably the first step in the reaction sequence from 24-methylenedihydrolanosterol to ergosterol (22). It has been postulated that the removal of the C-14 methyl group results in a C-14(15) double bond (1). The existence of an enzyme system in *U. maydis* capable of reducing this double bond was reported by Ragsdale (16).

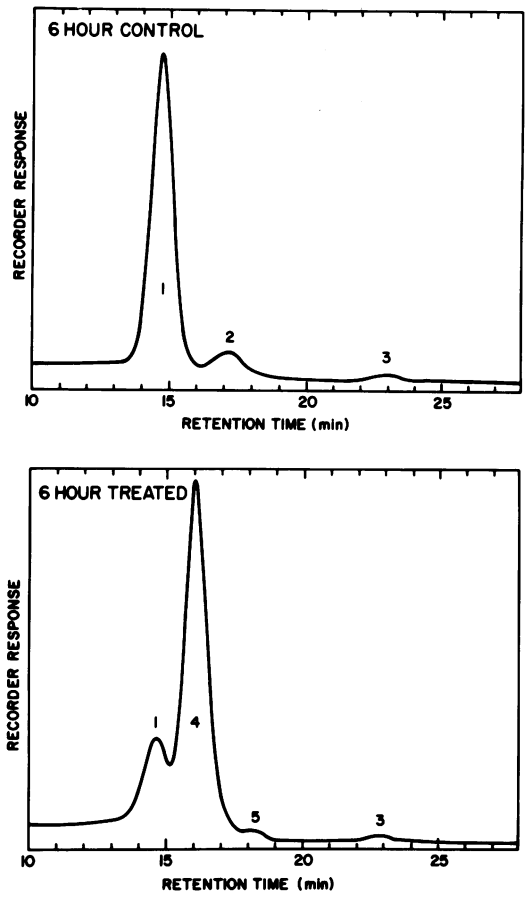

FIG. 6. *Gas-liquid chromatographic scans showing relative proportions of various free sterols in sporidia of 6-h azasterol-treated (0.1 μg/ml) and control cultures of U. maydis. Peaks represent: (1) ergosterol; (2) ergosta-5,7-dienol; (3) 24-methylenedihydrolanosterol; (4) ergosta-8,14-dien-3β-ol; (5) obtusifoliol.*

The accumulation of ergosta-8,14-dien-3β-ol in azasterol-treated *U. maydis* sporidia indicates that this enzyme system is the one selectively inhibited by the azasterol.

It is interesting that ergosta-8,14-dien-3β-ol is the only sterol intermediate that accumulates in significant quantities in treated sporidia, in view of the fact that inhibitors of C-14 demethylation such as triarimol (16) and triforine (22) lead to a large accumulation of the C-4,C-14 methyl sterols (obtusifoliol and 24-methylenedihydrolanosterol) and a gradual buildup of 14α-methylergosta-8,24(28)-dienol. Our data indicate that once the C-14 methyl is removed, C-4 demethylation proceeds rapidly even though the resulting C-14(15) double bond is not reduced. This is in contrast to the slow removal of the C-4 methyl groups when C-14 demethylation is inhibited (16, 20, 23). It is evident, therefore, either that C-14 methyl sterols are not good substrates for C-4 demethylation or that C-14 demethylation inhibitors also inhibit C-4 demethylation. There is evidence that the former is the case. Kato and Kawase (14), using cell-free homogenates from yeasts, demonstrated that the removal of the C-4 methyls from 14-desmethyl lanosterol was not impeded by the C-14 demethylation inhibitors Denmert and triarimol. Slow C-4 demethylation was also noted in *S. cerevisiae* mutants lacking the capacity for C-14 demethylation (23), again suggesting that C-14 methyl sterols are not ideal substrates for C-4 demethylation. In this case the mutant may also lack the normal C-4 demethylase; therefore, the slow C-4 demethylation may result from an induced alternate pathway.

The failure to remove the C-14 methyl or to reduce the C-14(15) double bond does not affect the C-24 alkylation (11, 17, 23). This is surprising in the case of *S. cerevisiae*, because this reaction normally occurs late in the ergosterol biosynthetic pathway (9). However, *S. cerevisiae* mutants lacking the ability to remove the C-14 methyl and *U. maydis* sporidia treated with C-14 demethylation inhibitors fail to reduce the 24-methylene group (20, 23). On the other hand, *U. maydis* and *S. cerevisiae* cells treated with the azasterol readily reduce the 24-methylene group (73). Thus it is evident that once C-14 demethylation occurs, the 24-methylene group is rapidly reduced. The failure to insert the C-22(23) double bond in the side chain, to shift the double bond at C-8(9) to C-7(8), and to insert a double bond at C-5(6) may result from direct inhibition by the azasterol, but more likely the C-14(15) double bond must be reduced before this reaction sequence can take place.

Results of this study are consistent with the idea that toxicity of the azasterol results from an inhibition of ergosterol biosynthesis, which leads to membrane damage because of the lack of an appropriate sterol for membrane structure. Ergosta-8,14-dien-3β-ol, the sterol that accumulates in treated sporidia, may not be released from the carrier protein, or if it does enter the membrane structure, the sterol may not interact effectively with the acyl hydrocarbon moieties of phospholipids due to the absence of axial hydrogens at C-9 and C-14 of the sterol nucleus (13).

The failure of the imazalil (and triarimol)-tolerant mutant of *A. nidulans* to respond differently from the wild type to the azasterol is consistent with the observations that imazalil (21) and the azasterol act at different sites in the sterol biosynthetic pathway.

The observed cross-resistance of the triarimol-tolerant *C. cucumerinum* mutant to the azasterol indicates that resistance in this organism has a different basis from that involved in imazalil (and triarimol) resistance in *A. nidulans*.

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